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THE INCORPORATION OF TRITIATED AMINO ACIDS BY CHROMOSOMAL PROTEINS IN THE HUMAN LYMPHOCYTE

BY

BARBARA CHERNICK

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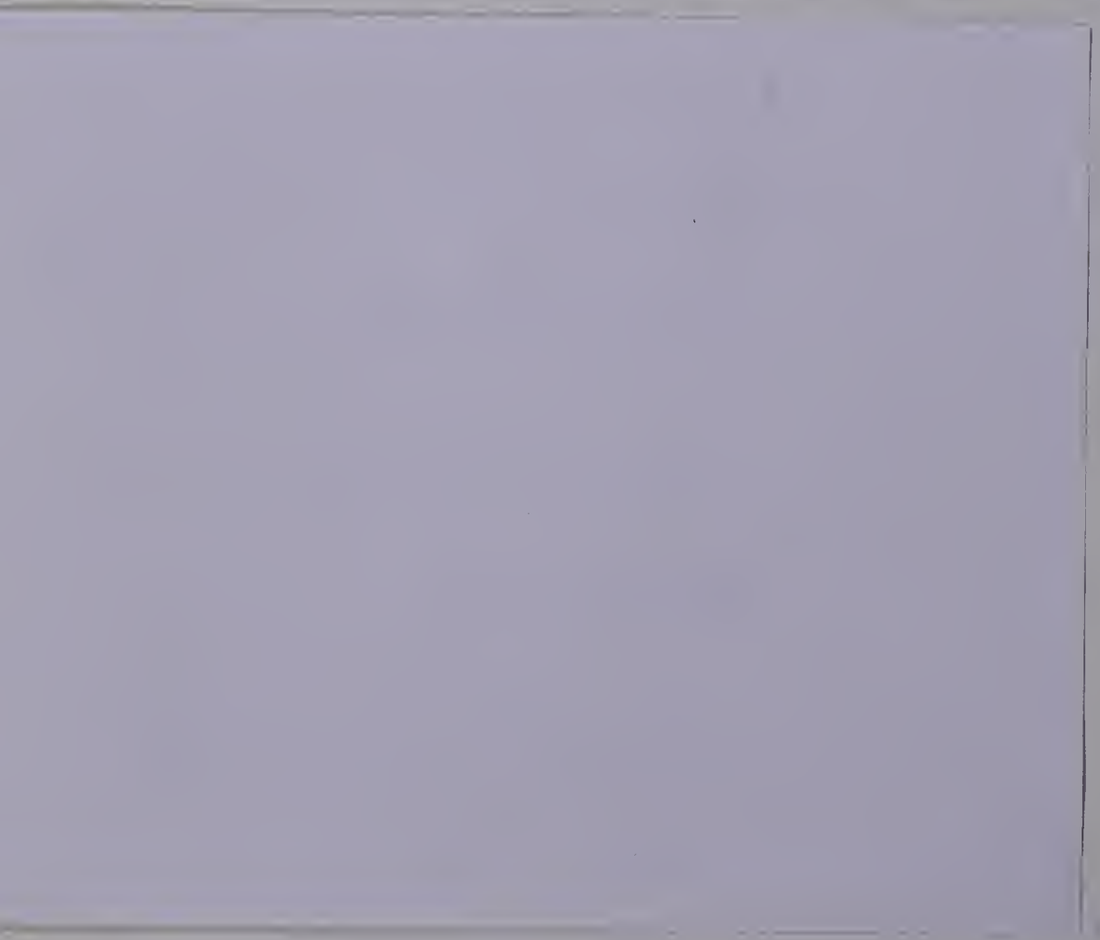

ABSTRACT

Chromosomal proteins were labeled with H^3-arginine and H^3-lysine. It was observed that acetic-ethanol fixation resulted in poorer preservation of chromosomal protein than did aqueous trichloroacetic-ethanol fixation or pre-treatment of acetic-ethanol fixation with formalin. Prolonged exposure to acetic-ethanol appeared to remove further protein in some instances. Acetic-ethanol fixation was used in early- and late-labeling experiments with both isotopes. From these studies it became apparent that not all chromosomes began and completed protein incorporation simultaneously. Nor was there absolute correspondence in labeling behaviour between isotopes. Chromosomes #3 and #19-20 were late in incorporation of both H^3-arginine and H^3-lysine. In the H^3-lysine experiments group #21-22 was also probably late in beginning incorporation while group #13-15 began earlier than the rest of the complement. Chromosomes #3 and #19-20 also appeared to finish labeling ahead of the other chromosomes with either amino acid. A late-labeling pattern for group #4-5 was observed only for H^3-arginine. Time studies of nuclear isotope incorporation revealed that significant amounts of amino acid were taken up in the first 6 hours of culture. However little label was found on metaphase chromosomes unless incubation with phytohemagglutinin had proceeded for the first 24-48 hours.

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INTRODUCTION.

To date, no studies of the pattern of protein synthesis in human leukocyte chromosomes have been reported. The pattern of DNA synthesis, however, has been investigated in some detail (Lima-de-Faria *et al.*, 1961; German, 1962; Gilbert *et al.*, 1962; Morishima *et al.*, 1962; Bader *et al.*, 1963; Schmid, 1963; Kikuchi and Sandberg, 1964; Bianchi and de Bianchi, 1965). Using tritiated precursors and autoradiography, these authors have demonstrated that DNA replication is asynchronous among non-homologous chromosomes, between homologues and chromatids, and among segments of the same chromosome. The purpose of the present study is to establish whether or not a pattern of protein synthesis exists among the chromosome groups. The discovery of a predictable sequence of labeling would lay the groundwork for a more detailed analysis of protein incorporation between homologues and chromatids and among segments of the same chromosome.

The chromosomal proteins investigated are those surviving acetic-ethanol fixation. Other fixative combinations were also evaluated for capacity to preserve chromosomal proteins *in situ*. It is hoped that the results will lead to the development of a fixative superior to acetic-ethanol in this respect.

Studies were also undertaken to determine when

isotope first entered the leukocyte nucleus, and whether this event also marked the onset of incorporation of protein onto the chromosomes.

The chromosomal proteins consist primarily of a basic group called histones, and a non-histone acidic group. At least one enzyme, phosphatase, has also been shown to be associated with mitotic chromosomes in chick cultures (Willmer, 1942). Histones are soluble in dilute acid (Kossel, 1921) and, unlike alkali-soluble acidic proteins, they contain virtually no tryptophan (Stedman and Stedman, 1943a). Because the specific arrangement of nucleic acids and proteins in the chromosome is as yet undetermined, several models have been proposed. Taylor (1963) has suggested that DNA helices are joined by some type of linker which, according to Bendich et al. (1963), may be acidic proteins. Mirsky and Ris (1947, 1950) regarded the acidic proteins as a core surrounded by DNA and histone. The acidic proteins do seem to be essential to the structural integrity of the chromosome as their removal alters chromosome morphology while removal of histone does not (Mirsky and Ris, 1950). It is possible that histones form bridges between DNA strands and also between coils of the same strand in such a way as to promote coiling of the DNA (Zubay, 1964). Wilkins et al. (1959) have suggested that histones may fit in the deep groove of the DNA molecule. Although the proteins are generally bonded to DNA through salt links, Kirby (1957)

has presented evidence which may be explained by the presence of a number of co-ordinate bonds. Huang (1965) also reports the presence in pea embryo chromatin of histone bound to RNA.

The Stedmans (1943b) proposed that histones may be modifiers of gene function. Since then investigators have sought to demonstrate inhibition by histones of nucleic acid synthesis, protein synthesis and growth, and to show differences in histones among tissues and species. *In vitro* evidence for histone inhibition of RNA synthesis was first provided by Huang and Bonner (1962) using pea embryo chromatin. According to Skalka *et al.* (1966), the presence of histone alters the size and base ratio of the RNA molecule synthesized. Liau (1965) has reported that the particular concentration of histone applied to a system of isolated nucleoli can also influence the base ratio of the RNA. Histones applied to suspensions of nuclear ribosomes have blocked amino acid incorporation into ribosomal proteins (Frenster *et al.*, 1961). In a complete-cell system of cultured embryonic chick brain, low concentrations of histone induced LDH production and high concentrations repressed it (Goodwin and Sizer, 1965). The induction of α-ketoglutarate transaminase and tryptophan pyrrolase synthesis in rat liver by hydrocortisone was suppressed if histone was injected simultaneously with the hydrocortisone (Caffery *et al.*, 1964). Histones have also been shown to inhibit tumor progression (Stedman, 1944), embryonic development (Brachet, 1964), and

to exhibit bactericidal properties, (Hirsch, 1958). Changes in the quality of histone have been observed during spermiogenesis in the snail, squid Bloch, 1962), grasshopper (Bloch, 1964), and during embryonic development of the sea urchin (Taleporos, 1959) and chick (Agrell, 1965). Neelin and Butler (1961) have prepared a histone fraction which appears to be characteristic of the chicken erythrocyte and Johns and Butler (1962) have noted the absence of an arginine-rich component in wheat germ. Histones have also been shown to differ in quality among several species of mammals and between immature and adult animals (Neidle and Waelsch, 1964).

In view of its apparent role as a repressor of biological function, the distribution and labeling pattern of chromosomal histone should be investigated. In the human female it has been shown that one X - chromosome is genetically inactivated to some extent (Lyon, 1961), and this may be due to the presence of a different kind or quantity of histone. Berlowitz (1965) has shown that the genetically inert paternal chromosomes of the mealybug possess 2.6 times more histone than the genetically active maternal set. Whether this histone can be demonstrated in human mitotic chromosomes is debatable, as the possibility exists that much histone may be removed by the presence of acetic acid in the customary fixatives (De, 1961; Busch et al., 1964). Further, there is some evidence that protein (histone?) naturally

departs from the chromosome during metaphase (Jacobson and Webb, 1952).

The biological significance of the acidic proteins is not clearly understood. It is known that they comprise about half of the nuclear proteins (Busch, 1965a) and are present in the chromatin (Wang et al., 1950) as well as other nuclear subfractions. In the chromatin they are thought to be associated with RNA (Mirsky and Ris, 1947), DNA (Mirsky and Ris, 1950), and lipid (Wang et al., 1952). Mirsky and Ris (1950) observed a positive correlation between the amounts of 'residual' (acidic) proteins in their chromosome preparations and the amount of cytoplasm in the same cells, and they suggested, as did the Stedmans (1943a), that these proteins may be important in genetic transmission. The puffing phenomenon observed in Dipteran salivary gland chromosomes is related to cell differentiation (Busch, 1965b) and the puffs consist mainly of non-histone proteins associated with RNA (Clever, 1964). Steele and Busch (1963) have reported that in tumor-bearing rats the uptake of radioactive lysine into the acidic proteins was greater than into the histones in the normal livers while the reverse was true of the tumors. It has been shown that nuclear acidic proteins are characterized by a very high turnover (Busch, 1965a).

REVIEW OF LITERATURE.

1. Growth of leukocytes in culture.

The discovery that mononuclear leukocytes could be stimulated to division in culture by phytohemagglutinin was first reported by Nowell (1960). Mitoses of cultured peripheral blood were noted as early as 1915 but the addition of phytohemagglutinin greatly increases the number of these divisions (Mellman, 1965). Phytohemagglutinin is a protein extract of the red kidney bean, Phaseolus vulgaris, or the navy bean, Phaseolus communis (Li and Osgood, 1949). It had been used as early as 1909 to agglutinate red cells (Mendel, 1909) and it is also a leukoagglutinin (Hirschhorn et al., 1963). The latter property is related to the mitogenic capacity of phytohemagglutinin though hemagglutination is not (Hirschhorn et al., 1963). The white cells stimulated to division are thought to be the lymphocytes (Cooper et al., 1963).

Exposure to phytohemagglutinin results in synthesis of protein, RNA, and DNA. Cultured lymphocytes will incorporate radioactive amino acids almost immediately without phytohemagglutinin stimulation, but after two hours the uptake reaches a plateau in the absence of phytohemagglutinin (Hirschhorn et al., 1963). The protein produced in either case, at least up to 24 hours, is γ - globulin (Bach and Hirschhorn, 1963). Quaglino et al., (1962) also report the synthesis of dehydrogenases in

phytohemagglutinin-stimulated leukocytes. It has been shown (Pogo et al., 1966) that protein as measured by the uptake of C^{14}-alanine increases in the presence of phytohemagglutinin after less than 15 minutes, but that histone labeling is not appreciable until after 24 hours. These authors also noted that addition of phytohemagglutinin greatly increased the rate of histone acetylation which appeared to precede the increase in nuclear RNA synthesis. Torelli et al. (1966) have found that the incorporation of protein and RNA precursors had increased significantly by 6 hours. Since these syntheses proceeded together the authors postulate that activation, not formation, of messenger RNA is occurring. Killander and Rigler (1965) also report an increase in capacity to stain with acridine orange in lymphocytes exposed to phytohemagglutinin. This is interpreted as an increase in the number of DNA phosphate groups made available for staining. These authors also report the onset of RNA synthesis at 12 - 24 hours and DNA synthesis at 24 - 48 hours. Others also estimate that DNA replication in phytohemagglutinin-stimulated lymphocytes begins at 24 hours with a peak of mitoses at 72 hours (MacKinney et al., 1962; Cooper and Barkhan, 1961; Cooper et al., 1963).

2. Time of chromosomal protein synthesis.

The time of histone synthesis seems to correspond with DNA synthesis in several systems. This is evident in

phytohemagglutinin-stimulated lymphocytes in which Pogo et al. (1966) report histone synthesis only after about 24 hours in culture, which time corresponds with the onset of DNA synthesis as mentioned above. Littlefield and Jacobs (1965) conclude that DNA and histone syntheses are normally concurrent although cessation of DNA synthesis by 5-fluorodeoxyuridine still permitted incorporation of C^{14}-valine into histones. When DNA synthesis was allowed to continue, the rate of incorporation into histone increased, paralleling the rate of DNA synthesis. A similar situation occurs in Vicia faba when both syntheses increase after telophase to a maximum at prophase (Woodward et al., 1961). In Euplotes also, DNA and histone replication in the macronucleus apparently proceed together (Gall, 1959). Bloch and Godman (1955) interpret a positive correlation between the amounts of DNA and histone among cells of various rat tissues as indicative of a possible time relationship between the two syntheses. In onion root-tip nuclei Alfert (1959) reports that histone is synthesized during the S phase, which is confirmed by De (1961), who also found histone synthesis to be occurring at even a higher rate afterwards. Niehaus and Barnum (1965) report that DNA and histone are synthesized during the same period in regenerating rat liver, as did Evans et al. (1962) who noted that DNA and one of the histone fractions reached peak concentrations together just before the appearance of a maximum number of mitotic figures. McLeish (1959) concludes that synthesis of DNA and histone

(as measured by the Sakaguchi test for arginine) proceed close together, if not simultaneously in plant cell nuclei.

However, in other studies of regenerating rat liver using radioactive precursors, Holbrook et al. (1962) observed that the specific activity of all histone fractions reached a peak earlier than did DNA. Butler and Cohn (1963) have shown that DNA synthesis begins about 18 hours after partial hepatectomy, whereas histone increases after 5 hours reaching a maximum at 18 hours. In tissues consisting predominantly of interphase cells (liver and kidney) and of cells in division (regenerating rat liver, rat Walker carcinoma, ascites mouse tumor), it has been found that the ratio of histone to DNA exceeds 2 in the 'interphase' tissues but is considerably lower in 'dividing' cells (Umaña et al., 1964). The authors also noted an increase in this ratio with time of sampling of regenerating liver after partial hepatectomy. They concluded that if histone and DNA syntheses were synchronized these differences and changes would not exist. Seed (1966) objects to the use of regenerating liver in studies of this kind on the grounds that no account has been taken of the liver cells which are not undergoing replication and which may still have been affected by the treatment.

According to Cave (1966) the synthesis of acidic proteins occurs throughout interphase in the phytohemagglutinin-stimulated lymphocyte. In following the incorporation of

H^3-lysine into chromosomal proteins resistant to dilute hydrochloric acid, he found that synthesis was low during the G1 period and increased during the S phase to a maximum at its end. Cave concludes that because H^3-lysine incorporation extended into the G2 period, synthesis of this protein was probably not related to DNA synthesis. Prescott and Bender (1962) report a study of the growth cycle of HeLa cells and a Chinese hamster line of cells wherein protein synthesis was measured by the uptake of H^3-histidine. By mid-prophase the rate of incorporation had decreased significantly below the interphase level and by late telophase it had begun to increase again. The cells were fixed with acetic-ethanol so presumably some, if not all, of the histones had been removed and the observed incorporation involved mostly the acidic proteins.

Howard and Pelc (1951) conclude that DNA and protein syntheses occur simultaneously in bean root cell nuclei. This protein was considered to be non-histone as hydrochloric acid was used in the Feulgen staining procedure. In Tradescantia as well as bean root nuclei, De (1961) observed non-histone protein synthesis both during and after the DNA-synthetic phase.

3. Site of chromosomal protein synthesis.

There is disagreement among authors as to the site of synthesis of histone. Busch (1958) has suggested that the nucleus is probably involved as histones are virtually absent

from the cytoplasm. However, basic proteins were first reported in the cytoplasm of Tetrahymena in 1955 (Alfert and Goldstein). The alkaline fast green test for histones (Alfert and Geschwind, 1953) has indicated the presence of histone in the cytoplasm of the sea urchin egg which later disappears during embryogenesis (Taleporos, 1959). Histone-like proteins have also been isolated from the yolk platelets of mature amphibian oocytes as well as the cytoplasm of the young oocyte (Horn, 1960, 1962). Nuclei of oocytes and embryonic cells were fast-green negative until the onset of gastrulation. Basic proteins appear in young mollusc oocytes and these are apparently bound to cytoplasmic RNA (Davenport and Davenport, 1965). Cytoplasmic basic proteins have also been demonstrated in Strain L mouse fibroblasts (Whitfield, 1965). These were associated with RNA and were only demonstrable when the cells were not treated with alcohol and xylol before mounting. Biochemists have indicated that basic proteins with some similarities to histone are bound to ribosomal RNA in cytoplasm of rat liver cells (Crampton and Petermann, 1959). During spermiogenesis in the grasshopper the appearance of arginine-rich histone is first detected in a cytoplasmic layer surrounding the nucleus (Bloch and Brack, 1964). This layer contains ribosome-like particles, suggesting to the authors that histone originates in the cytoplasm where it is associated with RNA, and later migrates to the nucleus to combine with DNA.

Evidence for ribosomal participation in histone synthesis is suggested by the blocking of histone synthesis by puromycin reported for Ehrlich ascites tumor cells (Schweiger et al., 1964). However, ribosomes are reported to occur in the nucleus as well as the cytoplasm in certain cell types (Allfrey, 1963). Birnsteil and Flamm (1964) and Birnsteil et al. (1964) claim to have isolated histone-type protein as well as residual (or acidic) proteins from the nucleolus. Tobacco cells and isolated pea nucleoli were pulse-labeled with C^{14}-amino acids. It was observed that the nucleolus incorporated radioactivity into both histone and non-histone fractions. This seems to be rather conclusive evidence that at least some histone synthesis is nucleolar. De (1961) also provides autoradiographic evidence in plant cell nuclei that nucleoli are synthesizing histone and that nucleolar synthesis precedes incorporation of tracer by the chromatin and cytoplasm.

There are several reports indicating that residual or acidic protein synthesis is also nuclear. However there is little evidence as to the exact site of synthesis of those acidic proteins contributing to chromosomal structure. Birnsteil and Flamm (1964) have shown that the nucleolus incorporates C^{14}-amino acids before other nuclear organelles and that the nucleolar non-histone protein incorporates particularly high radioactivity. However this fraction is almost identical to ribosomal protein

in amino acid composition, suggesting a site of synthesis of ribosomal components. Patel and Wang (1965) report that after extraction of nuclear ribosomes from isolated calf thymus nuclei the remaining complex of nucleolar components, RNA, DNA, histone and non-histone protein, can incorporate C^{14}-tryptophan which is considered to be specific for acidic proteins. This process was DNA-dependent and was stimulated by histone removal. In a study of the incorporation of S^{35}-methionine into Drosophila salivary gland chromosomes, Sirlin (1958) observed a concentration of autoradiographs over the nucleolus, but not over the nucleolus-associated DNA. Acidic protein synthesis was indicated as the preparations had undergone hydrolysis with dilute hydrochloric acid which would remove histone. The incorporation of H^3-tryptophan into plant cell nuclei was shown by De (1961) to occur in the nucleolus initially, followed by incorporation into the chromatin and cytoplasm.

4. Disappearance of protein from the chromosomes at metaphase.

The proteins do not seem to be permanently associated with chromosomes in the same manner as DNA. Prescott (1963) has cultured Chinese hamster fibroblasts with several H^3-amino acids and, using autoradiography, has followed the segregation of chromosomal protein at 1, 2, 3 and 4 divisions after labeling. Between the first and second divisions the reduction in radioactivity was greater than could be accounted for by simple chromosome division and by the fourth

division the chromosomes were no longer labeled. There was an equal distribution of isotope between the two chromatids at the first and second division. A similar loss of radioactivity was observed in successive mitoses in Vicia faba (Prensky and Smith, 1964).

In staining experiments with the May-Grunwald and Giemsa reagent Jacobson and Webb (1952) found that ribonucleoprotein was apparently shed from mammalian chromosomes during anaphase. Similar findings are reported by Woodward et al. (1951) for mitosis in Vicia faba. Sims (1965) observed that nuclear proteins left the chromosome during prophase and prometaphase and returned to the nucleus in late telophase in rat tissues labeled in vivo with H^3-tyrosine. In Amoeba proteus it has been shown that radioactive nuclear proteins are released into the cytoplasm during late prophase (Prescott and Stone, 1965). These returned to the nuclei of late telophase and early interphase daughter cells. A higher histone /DNA ratio is reported in isolated nuclei of interphase-rich mammalian tissues than in tissues with a large number of dividing cells (Umaña et al. 1964).

The significance of this mobility of nuclear protein at cell division is not understood. It may indicate protein involvement in the coiling and uncoiling of the chromosome at this stage. In studying the effects of inhibitors of RNA and protein synthesis during meiosis, Kemp (1964) concluded that

both syntheses are necessary during meiotic prophase to maintain the condensed state of the chromosome, as well as to ensure separation of homologous chromosomes and normal spindle behaviour.

5. Distribution of protein on the chromosomes.

Prescott and Stone (1965) observed that the pattern of amino acid incorporation onto hamster chromosomes followed that of H^3-thymidine. These authors include formaldehyde in their acetic-ethanol chromosome preparations and so are probably preserving more histone than with acetic-ethanol alone.

The distribution of protein on the chromosomes has been studied in several species of Diptera. Pelc and Howard (1956) report autoradiographs of uneven intensity along the larval salivary gland chromosomes of the Diptera. There were insufficient grains to determine whether or not there were concentrations over the bands. The glands were squashed with 45% acetic acid which may have removed all or, at least some, of the histones. Sirlin and Knight (1958) found that incorporation of S^{35}-methionine into Drosophila salivary gland chromosomes resulted in a uniform distribution of silver grains throughout the chromosome complement with a slight reduction over the heterochromatin of the chromocenter. Both chromatids had a similar labeling pattern (Sirlin and Knight, (1960). The pattern persisted after withdrawal of tracer or treatment with potassium cyanide to unfold the

chromosomes. Again, acidic proteins are indicated because of hydrolysis treatment. Incorporation of C^{14}-phenylalanine into the salivary gland chromosomes of Rhynchosciara angelae is apparently increased in the areas of the puffs, corresponding with increased incorporation of DNA precursors (Ficq et al., 1958). Acetic acid was employed in the fixative. A close correspondence between concentrations of histone and DNA in the Drosophila salivary gland chromosome has been reported by Horn and Ward (1957) and Swift (1964).

Pelc and Howard (1952) have made a detailed study of the distribution of silver grains on the M chromosomes of Vicia faba after incorporation of inorganic S^{35}. The autoradiographic pattern was reproducible among M chromosomes of different cells and was undoubtedly due to acidic protein synthesis because the preparations had undergone Feulgen hydrolysis with 1 N hydrochloric acid.

Similar studies of the distribution of autoradiographs within individual chromosomes and between groups of chromosomes treated with radioactive amino acids have not, to my knowledge, been reported for the human lymphocyte.

MATERIALS AND METHODS.

1. Blood culture

Whole peripheral blood from the same healthy female was used in all experiments. It was cultured by the microtechnique of Arakaki and Sparkes (1963). Blood was drawn from a finger prick into a heparinized capillary pipet and expelled into a 25 ml. sterile plastic culture flask (Falcon Plastics). The latter contained 5 ml. of Eagle's (Diploid) medium (General Biochemicals) with 10% calf serum (Difco), 100 units/ml. of penicillin G and 100 µg./ml. of streptomycin sulfate (Difco). If lymphocyte stimulation was desired, 0.1 ml. phytohemagglutinin-M (Difco) was also added. In experiments designed to determine the earliest period of isotope incorporation into nuclei, media and culture flasks were pre-warmed to 37°C before setting up the cultures.

Cultures in which isotope was to be removed and replaced with 'cold' medium were initiated with cultures receiving no isotope. At changing time the medium from the 'cold' cultures was added to the isotope-treated cells which were first washed twice in complete medium. The cultures were also transferred to new flasks at this time.

2. Isotopes

The isotopes studied were H^3-L-lysine (Schwartz BioResearch Inc., sp. act. 230 mc/mm) and H^3-L-arginine (Schwartz BioResearch Inc., sp. act. 350 mc/mm). These were

added to the blood cultures to give a final concentration of 0.5 μc/ml. or 1.0 μc/ml.

3. Smear preparations

Smear preparations of interphase leukocytes were made by centrifuging off the growth medium, rinsing twice in calf serum and resuspending in a minimum of calf serum. A drop was placed on a slide and smeared with the edge of another slide. Slides received no further treatment except in the fixation experiments.

4. Chromosome preparations

A maximum number of cells in metaphase were obtained by adding colcemid (CIBA) to the cultures 3 - 4 hours before harvest to a final concentration of 0.2 μg/ml. Cells were harvested at 72 hours after culture initiation except where the isotope-containing medium was replaced with 'cold' medium sometime during the culture interval. In this case harvesting was delayed 6 hours. When hypotonic treatment was used to spread the chromosomes, all but 0.5 ml. of culture medium was removed and 1.5 ml. of distilled water was added and the cells resuspended. Two ml. of distilled water were added in the formalin experiments. Hypotonic treatment lasted 10 minutes including centrifuging time.

5. Fixation

These included (1) Clarke's acetic-ethanol (Baker, 1958a) in the proportions 1 part glacial acetic acid to 3 parts 95% ethanol. Freshly prepared fixative was added to

the button of cells remaining after removal of the hypotonic medium. The cells were suspended in this and allowed to stand 1/2 to 1 hour unless otherwise specified. The fixative was replaced twice with a fresh mixture and several drops of the final cell suspension were placed on a clean slide and air-dried or flamed to ignite the fixative. (2) Aqueous trichloroacetic-ethanol: 100 grams of trichloroacetic acid were dissolved in 100 ml. of distilled water. One part of this was added to 4 parts of 95% ethanol. Cells to be fixed were suspended in 0.1 ml. of medium and the fixative added forcefully, with rapid and immediate pipeting to break up the coagulum. Fixation lasted 1/2 to 1 hour and the fixative was replaced twice. One or two drops of cell suspension were placed on a clean slide and the fixative ignited, or the cells were allowed to settle. Then both flamed and unflamed slides were placed in 95% ethanol to remove the trichloroacetic acid and water. (3) Formalin pre-treatment of acetic-ethanol preparations (Prescott and Bender, 1963): cells and medium were drawn up into a capillary pipet and an equal volume of 10% neutral formalin added. Then after 15 seconds a volume of acetic-ethanol equal to that already in the pipet was drawn up and the mixture expelled onto a slide wet with acetic-ethanol. The cells were allowed to settle on the slide a few seconds and then the slides were placed in 95% ethanol to remove the formalin and acetic acid.

6. Autoradiography

Slides were dipped in NTB_3 liquid emulsion (Kodak) which had been warmed to 44 - 46°C. This was done in the dark or with the aid of a lamp at least 3 feet away with a red Wratten Series 2 filter. The slides were allowed to dry 20 - 30 minutes and were then stored in light-tight boxes at 4°C for 4 or 7 days. They were then developed for 2 minutes in D-11 (Kodak) developer, fixed 4 minutes in acid fixer (Kodak), and rinsed in running water 15 - 20 minutes.

7. Staining

Nuclei and chromosomes were stained through the developed emulsion with Gurr's Improved Giemsa "R 66". 1 ml. of stain was added to 18 ml. of tap water and the slides were stained for 2 - 4 minutes, rinsed in distilled water and immediately blotted dry. Coverslips were not affixed.

8. Microscopy

Grain counts were made under oil immersion of either a Zeiss microscope at a magnification of 1600 or a Leitz microscope with a magnification of 1000. Lymphocytes of all sizes were assessed, as described by Diggs et al. (1954). Counts over metaphases included only those grains actually over or touching a chromosome. The chromosomes of a karyotype were identified through the microscope and the position of the silver grains was recorded on prepared diagrams.

9. Statistical analysis

In all statistical tests the 5% level was accepted as significant.

In the studies of isotope incorporation into interphase nuclei, the number of grains over each of 100 nuclei was recorded for each treatment and its control. The randomized analysis of variance (Goulden, 1952a) was used to test for significant differences between the means.

In comparisons of the various chromosome fixatives, the grains over a variable number of metaphases were recorded and the number of metaphases appears in each table in the Results and Discussion. The unpaired "t" test (Goulden, 1952b) was used to test for significant differences between the means.

The use of the "t" test or the analysis of variance assumes a normal distribution of variates (Snedecor, 1956a). However "counts of variables tend to be distributed in Poisson fashion with the variance proportional to the mean" (Snedecor, 1956b). Consequently each count was transformed to $\sqrt{X}$ (or the $\sqrt{X+1}$ where zeros appeared) before these tests were applied.

A statistical approach was employed to determine whether or not the chromosome groups began and ended isotope incorporation together. It was expected that metaphases with high grain counts would have been incorporating longer, and also that the grains over them would tend to random

labeling assuming uniform protein distribution. For a chromosome 4 combinations of results are possible in an experiment:

Metaphases with a	1	2	3	4
A. High Count	random	random	non-random	non-random
B. Low Count	random	non-random	random	non-random
Interpretation:				
Relation to time of labeling of other chromosomes	in phase	out of phase	out of phase	out of phase
Protein distribution along chromosome	uniform	uniform	uniform or non-uniform	uniform or non-uniform

It will be seen that to establish that labeling is asynchronous, combination #2 must be obtained. #3 and #4 do not exclude the possibility that non-random counts may be due to uneven protein distribution among chromosomes.

The observed grain counts for any chromosome group were compared with those expected on the basis of random distribution, using the chi-square test (Snedecor, 1956a). Randomly-occurring grains should be present in quantities proportional to the relative chromosome length. Relative chromosome lengths for the human karyotype were calculated from the average of the ranges presented by Puck (1962). The two chi-square classes consisted of (1) the total grains found over a particular chromosome group, and (2) the total grains over all the other chromosomes of the metaphase. Chromosomes of the #6-X-12 group and the #16-17-18 were not considered. No attempt was made to distinguish between the members of the #4-5 group, the #19-20 group, or the #13-14-15.

RESULTS AND DISCUSSION.

1. Fixation studies

A series of trials were undertaken to determine to what extent prolonged exposure to acetic-ethanol would remove chromosomal protein. Cultures were incubated with isotope for the full 72 hours and then stored in acetic-ethanol for various intervals. Metaphase counts appear in Table 1.

TABLE 1

Average Grain Counts Per Metaphase in Cells exposed for various intervals to acetic-ethanol.

Exp. No.	Isotope	Length of Time in Fixative	Number of Metaphases	Average Grain Count
1.	H^3-arginine	2 min.	8	32.5
		3 hours	16	33.6
		19 hours	16	23.0
2	H^3-arginine	30 min.	16	86.5
		1 hour	16	100.0
3	H^3-lysine	30 min.	35	34.8
		1 hour	41	26.0
		25 hours	22	26.0
		49 hours	14	22.1
4	H^3-~~DL~~ lysine	30 min.	29	19.4
		1 hour	25	19.4
		24 hours	32	24.0
		48 hours	8	18.5

Increasing exposure to acetic-ethanol resulted in loss of label from the chromosomes only in experiment #3. The difference between 1 or 25 hours and 49 hours is statistically significant. Of course it is possible that in the other cases protein removal was instantaneous. However, if this were true one would expect a difference at least between the 2 minute and 3 hour fixation periods in experiment #1.

The Stedmans (1950) calculated that 26.5% of the total histone had been removed from isolated liver nuclei by 30% acetic acid in ethanol. Two experiments were carried out to see whether a similar loss of labelled protein occurred in interphase lymphocyte nuclei. Lymphocytes were cultured the full 72 hours in the presence of isotope and fixed 1 1/2 hours in acetic-ethanol or aqueous trichloroacetic-ethanol. Both fixatives were ignited on the slide. The cells were not pretreated with hypotonic. The results appear in Table II.

Table II

Average grain count per interphase nucleus of lymphocytes cultured with isotope and fixed by two methods.

	H^3-Arginine	H^3-lysine
Acetic-ethanol	19.4	24.0
Aqueous Trichloroacetic-ethanol	25.0	28.1

The aqueous trichloroacetic-ethanol preserved significantly more label in the nuclei than did the acetic-ethanol with both isotopes. This fixative was developed in an effort to precipitate all histone fractions; Daly and Mirsky (1954) observed that 13% trichloracetic acid was required to precipitate lysine-rich histones while 7 1/2% sufficed for the arginine-rich. The acidic proteins were probably preserved as they are only soluble in alkali, (Stedman and Stedman, 1943). These results support the Stedman's findings (1950) although this experiment does not define the type of nuclear protein lost.

Prescott and Bender (1963) pretreated mitotic hamster fibroblasts for 60 seconds with 10% neutral formalin before a short exposure to acetic-alcohol in an effort to retain protein on the chromosome preparations. They claim that this was successful and are corroborated by Rabinowitch in the discussion of this meeting who found that post-fixing amoebae in formalin reduced protein loss in water washing after acetic acid fixation. Consequently an attempt was made to evaluate the effect of formalin pretreatment of mitotic lymphocyte cells. These were cultured for 72 hours in the presence of isotope.

After hypotonic treatment half of the cells were pre-treated with neutral formalin for 15 seconds and then dropped with acetic-ethanol onto a fixative-treated slide as described in the Materials and Methods. The other half of

the cells were treated in precisely the same way but with the omission of the formalin treatment. Total time of exposure in each fixative treatment to acetic-ethanol did not exceed 5 minutes. The metaphases obtained either way were not spread sufficiently to perform a karyotype analysis, but were adequate for comparative grain counts. Undoubtedly there would be some interfering radioactivity from the cytoplasm of the poorly-spread cells but this was considered to be equivalent in both preparations. The results appear in Table III. For both isotopes, the formalin pre-treatment resulted in significantly higher retention of protein as measured by grain count. These results agree with those of De (1961) who found that a fixation consisting of alcohol, glacial acetic acid and formalin was superior to acetic-ethanol in preserving proteins of the chromatin.

TABLE III

Average grain count per metaphase in lymphocytes cultured with isotope and fixed by two methods.

	H^3-arginine	Number of metaphases	H^3-lysine	Number of metaphases
Acetic-ethanol	12.5	19	8.6	30
Formalin-acetic-ethanol	16.1	19	23.1	30

According to Baker (1958c), acetic acid precipitates nucleoproteins due to the action of the acetate ion at a pH less than 4.4. I have found that the customary 1:3 acetic-ethanol mixture has a pH of about 2.75. The acetate ion is also said to separate DNA from combination with protein with immediate precipitation. Perhaps in the present experiments this precipitated protein was lost from the chromosomes by pipetting and centrifuging during changes of fixative. Or the higher grain counts in the formalin preparations may be the result of preservation of chromomally-associated enzymes. These experiments do not define the type of protein being observed. There is some evidence from other sources that the protein in question may be histone. De (1961) found that 1:3 acetic-ethanol removed histone from mitotic chromosomes of onion root cells and Busch et al., (1964) report a similar finding for fixatives containing 30-50% acetic acid. These investigators do not show whether or not all the histone is removed. It probably is not; I have been able to stain acetic-ethanol preparations of chromosomes by the alkaline fast-green technique for histone (Alfert and Geschwind, 1953), although the chromosomes bound insufficient dye to photograph. Formaldehyde is said to cross-link neighboring peptide chains through methylene bridges between various residues such as amino, amido, imino, peptide, guanidyl, hydroxyl, carboxyl, sulfhydryl and aromatic rings (Pearse, 1960). This action

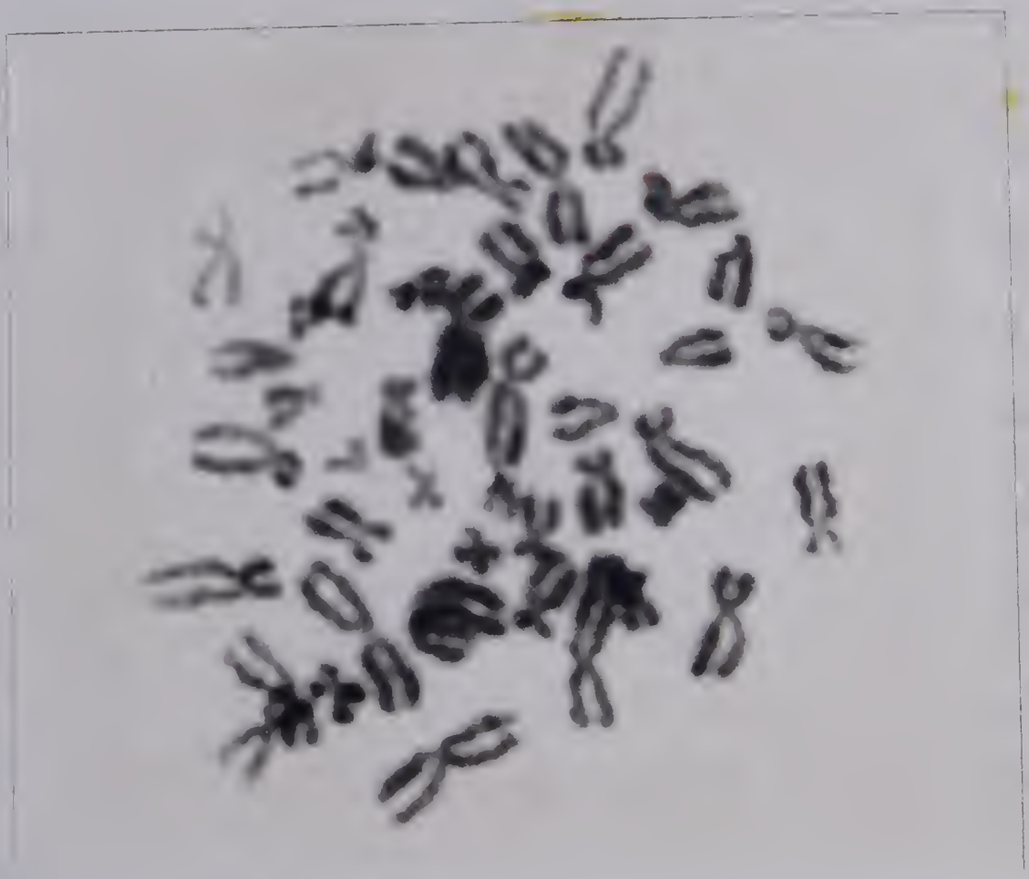

Figure 1.

Chilled trichloroacetic-ethanol fixation.

Hypotonic pre-treatment.

was probably responsible for preserving chromosomal protein in my experiments. Whether or not such a brief exposure to formalin is 100% effective is unknown, but it is highly unlikely (Pearse, 1960). At any rate it would appear that acetic-ethanol does remove some chromosomal protein. If this is nucleoprotein then the precipitating action of acetic acid may not have been complete, or else precipitation did not hold the protein in situ.

It was found that aqueous trichloroacetic-ethanol could be used to prepare recognizable karyotypes as in Figure 1. The yield of good metaphases was low. Subsequent studies indicated that fixation periods of 1/2 to 1 hour's duration produced optimally spread metaphases, although these were few if hypotonic treatment was not used. Non-aqueous trichloroacetic-ethanol (1:4 w/v) was also tested but the extra alcohol resulted in severe cell shrinkage.

A series of trials were conducted next to compare aqueous trichloroacetic-ethanol with acetic-ethanol for capacity to preserve chromosomal protein. Phytohemagglutinin-stimulated lymphocytes were cultured for the usual 72 hours with H^3-lysine and half of the cultures were treated with each fixative without previous hypotonic treatment. The results are presented in Table IV A. There was no significant difference in grain count. Omission of hypotonic resulted in

poorly-separated chromosomes fixed with acetic-ethanol so the experiment was repeated allowing a 10 minute hypotonic treatment for cells to be prepared with this fixative. Again the fixatives did not differ significantly (Table IV B) although the acetic-alcohol average is considerably lower.

TABLE IV

Average grain count per metaphase in lymphocytes cultured with H^3-lysine and fixed by two methods. Fixatives ignited.

	A. NO HYPOTONIC		B. HYPOTONIC FOR ACETIC-ETHANOL	
	Average Grain Count	Number of Metaphases	Average Grain Count	Number of Metaphases
Aqueous trichloro-acetic ethanol	27.0	13	38.4	13
Acetic-ethanol	29.2	15	31.4	13

There are probably several reasons for this. The number of metaphases evaluated is necessarily low as it was difficult to obtain large numbers of well-spread metaphases with the aqueous trichloroacetic-ethanol. Consequently the 't' value required for significance is high. There was also considerable variability among counts which resulted in a large error term for testing the differences between means. At least part of this variability was probably due to the fact that both fixatives were ignited to improve chromosome spreading. This resulted in a 'melted' appearance of some metaphases in the aqueous trichloroacetic-ethanol preparations,

and it was suspected that this treatment may remove DNA with attached protein. Schneider (1945) has shown that 5% trichloroacetic acid at 90°C for 15 minutes suffices to remove DNA from fixed preparations. Therefore, the above experiment was repeated with H^3-arginine, allowing the cells to air dry on the slides. The acetic-alcohol fixative was preceded by hypotonic treatment. This time the grain counts were significantly lower for the aqueous trichloroacetic-ethanol preparations (Table V) and some metaphases still appeared to be melted.

TABLE V

Average Grain Count per metaphase in lymphocytes cultured with H^3-arginine and fixed by two methods. Fixatives not ignited.

	Average Grain Count	Number of Metaphases
Aqueous trichloroacetic-ethanol	12.0	28
Acetic-ethanol	38.7	26

Consequently the experiment was repeated for both isotopes chilling the aqueous trichloroacetic-ethanol to 4°C and also the 95% ethanol used to rinse the slides. Both treatments were preceded by 10 minutes in hypotonic. The chilling resulted in a minimum of melted cells or metaphases and the comparison appears in Table VI. The aqueous trichloroacetic-ethanol treatment has a higher grain count than the acetic-ethanol for both isotopes although the

difference is significant only for H^3-lysine.

TABLE VI

Average grain count per metaphase in lymphocytes cultured with isotope and fixed by two methods. Chilled trichloroacetic-ethanol.

	H^3-arginine	Number of Metaphases	H^3-lysine	Number of Metaphases
Aqueous Trichloroacetic-ethanol	98.3	9	16.3	26
Acetic-ethanol	94.5	9	10.2	26

Evidently the aqueous trichloroacetic-ethanol is superior to acetic-ethanol in preservation of chromosomal protein under the right conditions. Whether the protein involved is histone or of enzyme nature is undetermined. Experiments are now in progress to combine formalin pre-treatment with aqueous trichloroacetic-ethanol fixation. If adequate metaphase preparations can be obtained, this method will be recommended for studies of the distribution of total protein on the chromosome.

2. Time of Nuclear Incorporation.

As part of an investigation of the temporal relationship between nuclear and chromosomal incorporation of H^3-arginine and H^3-lysine, cultures grown in the presence of isotope were harvested at 24, 48 and 72 hours after initiation of the culture. Control cultures received no phytohemagglutinin. The average grain count per nucleus presented in Table VII A are significantly higher in the

phytohemagglutinin-stimulated cells on all 3 days for both isotopes. Treated cultures showed a steady increase in isotope incorporation from 24 to 72 hours. Evidently protein synthesis was initiated some time in the first 24 hours so the experiment was repeated, harvesting at 8, 16, and 24 hours (Table VII B). Again the treated cultures incorporated significantly more nuclear label than did the controls, with an increase from 8 to 24 hours. In the next trial similar cultures were harvested at 2, 4 and 6 hours (Table VII C). The lymphocytes exposed to H^3-arginine approached a significant increase over the control by 2 hours and were significantly higher at 4 and 6 hours. In the H^3-lysine series, significant nuclear uptake in treated cells was not apparent until the 6th hour.

TABLE VII

Average grain count per nucleus in lymphocytes harvested at various intervals, treated and untreated with phytohemagglutinin.

	H^3-arginine Time of harvest Hours			H^3-lysine Time of harvest Hours		
A.	24	48	72	24	48	72
With phytohemagglutinin	3.7	4.2	13.8	3.7	6.2	8.9
Without phytohemagglutinin	0.7	1.2	1.4	0.6	1.0	0.7
B.	8	16	24	8	16	24
With phytohemagglutinin	1.6	3.2	4.4	1.8	1.1	4.8
Without phytohemagglutinin	0.8	0.8	1.2	0.6	0.1	0.8
C.	2	4	6	2	4	6
With phytohemagglutinin	0.7	0.9	2.1	0.1	0.3	0.3
Without phytohemagglutinin	0.4	0.5	0.9	0.1	0.2	0.2

In the 2, 4 and 6 hour series counts were also made over the cytoplasm of the same cells (Table VIII). In H^3-arginine treated lymphocytes significant incorporation over the control is not evident until after 6 hours. Cytoplasmic incorporation was not significant at any of these periods in the H^3-lysine cultures.

TABLE VIII

Average cytoplasmic grain count per lymphocyte in cultures harvested at various intervals, untreated and treated with phytohemagglutinin.

	H^3-arginine Time of harvest Hours			H^3-lysine Time of harvest Hours		
	2	4	6	2	4	6
With phytohemagglutinin	0.4	0.4	1.2	0.1	0.2	0.2
Without phytohemagglutinin	0.3	0.3	0.6	0.2	0.1	0.2

Nuclear grain counts undoubtedly included disintegrations originating in the thin layer of cytoplasm overlying the nucleus, which is why cytoplasmic counts were made. It is interesting that significant incorporation into the nucleus precedes that in the cytoplasm with both isotopes. This may represent nucleolar synthesis of non-histone protein as demonstrated in tobacco and pea nuclei by Birnsteil *et at*. (1964). Histone synthesis in the phytohemagglutinin-stimulated lymphocyte does not become 'appreciable' until after 24 hours' incubation according to Pogo *et al*. (1966). Bach and Hirschhorn (1963) have shown that the early increase in protein incorporation in phytohemagglutinin-stimulated lymphocytes represents γ-globulin synthesis. The possibility that at least some of this may occur in the nucleus cannot be excluded as Kirkham and Thomas (1953) have isolated globulins from calf thymus and calf liver nuclei, though they do not

specify whether or not these are γ-globulins.

While counting silver grains it was noticed that a high percentage seemed to occur in a single spot just inside the nuclear membrane. To verify this observation nuclei from the 4-hour H^3lysine cultures were classified as to the location of this grain. It appeared just inside the nuclear membrane in 21 out of 38 nuclei from phytohemagglutinin-stimulated cultures, and in 20 out of 38 nuclei in the controls. It is tempting to suggest that this grain may cover the late-replicating female X-chromosome demonstrated in human lymphocytes by Morishima (1962). During interphase this chromosome is thought to be partly genetically inactive (Lyon, 1961) and supposedly it constitutes the condensed 'sex chromatin' occurring at the nuclear periphery in cells of various female tissues (Barr and Bertram, 1949). It is not demonstrable in human lymphocytes by the usual basic stains because the entire nucleus stains too deeply. However, Ribas-Mundo (1966) has shown that in leukocyte cultures labelled with H^3-thymidine some cells became heavily labelled in a spot next to the nuclear membrane. This spot did not coincide with the position of the nucleolus and he supposed that it corresponded with the female X-chromosome which replicates its DNA later than the rest of the complement. In my preparations it may be that this chromosome is incorporating H^3-lysine out of phase with other nuclear components. Lysine-rich histones are reported to be

particularly inhibitory of RNA synthesis (Huang et al. 1964).

As the air-dried slides in these experiments received no further fixation it was suspected that some soluble proteins might be lost during the autoradiographic process. Consequently the 4- and 6-hour H^3-lysine experiments were repeated, treating half the slides with cold non-aqueous trichloroacetic-ethanol (1:4, w/v) for 15 minutes in the refrigerator (Table IX). Fixed phytohemagglutinin-stimulated lymphocytes had incorporated significantly more isotope than fixed controls only after 6 hours. This corresponds with the results of H^3-lysine incorporation of Table VII C from the earlier unfixed trial. A comparison between fixed and unfixed cells treated with phytohemagglutinin indicates that the fixative preserves significantly more protein at both 4 and 6 hours. But there is no significant difference between fixed and unfixed controls at either time, suggesting that only the protein synthesized in response to phytohemagglutinin is extracted in the autoradiographic process. The latter employs aqueous solutions and a final water rinse as well as a dilute acid fixer. Commerford et al. (1963) have shown that water may be used to extract nucleohistone from calf liver nuclei and that this can happen in 20 minutes, but as mentioned earlier, little new histone has been synthesized by 6 hours. Acidic nucleo-proteins may perhaps be removed by the alkaline developer. However the globulins probably account for the bulk of the loss in isotope as these are the

main proteins first synthesized in response to phytohemagglutinin (Bach and Hirschhorn, 1963).

TABLE IX

Average grain counts per nucleus in lymphocytes cultured with H^3-lysine, harvested at various intervals, with and without fixation.

		Time of harvest, Hours	
		4	6
Fixed	With phytohemagglutinin	0.6	0.5
	Without phytohemagglutinin (control)	0.4	0.3
Unfixed	With phytohemagglutinin	0.1	0.1
	Without phytohemagglutinin (control)	0.1	0.2

Prescott and Bender (1962) and Baserga (1962) have used cold trichloroacetic acid treatment of their slides prior to dipping in autoradiographic emulsion to extract radioactive amino acids which were not incorporated into protein. To determine whether or not such treatment was necessary after the acetic-ethanol method of chromosome preparation, leukocytes were incubated the full 72 hours with H^3-arginine and chromosome preparations were made as usual. Half of the slides were placed in cold aqueous trichloroacetic-ethanol and maintained at 4°C for 15 minutes. They were then rinsed in cold 95% ethanol. The comparison appears in Table X. The two averages are not significantly different so no extraction procedure was adopted.

TABLE X

Acetic-ethanol chromosome preparations. Untreated and treated with cold aqueous trichloroacetic-ethanol.

	Average Grain Count	Number of Meatphases
Treated	60.5	32
Untreated	59.1	34

To determine whether or not the unfixed interphase nuclei evaluated for time of protein incorporation would really be comparable to cells processed for chromosome preparation, cultures incubated with phytohemagglutinin and H^3-arginine for the full 72 hours of culture were fixed in two ways. Half were smeared on slides as described in the Materials and Methods and half were treated with hypotonic and acetic-ethanol and air-dried on the slide as for chromosome preparations, Colcemid was not used. Nuclear grain counts appear in Table XI.

TABLE XI

Average grain counts over lymphocyte nuclei incubated with H^3-arginine and phytohemagglutinin and fixed in two ways.

Smear	23.0
Hypotonic and acetic-ethanol	24.0

The two means are not significantly different. This is an indication that the unfixed nuclei of the earlier experiments retained as much protein as cells processed for chromosome preparation.

Early addition of isotope H^3-arginine

Preliminary experiments indicated little chromosomal incorporation until after 48 hours' incubation with H^3-arginine. The observed and expected grain counts on the basis of random distribution from a culture exposed to isotope for the first 50 hours appear in Table XII. The chi-square value required for significance in all labeling experiments is 3.84.

A = Combined counts of all 19 metaphases, averaging 25.6 grains per metaphase.

B = Combined counts of metaphases, averaging 20.1 grains, range 14-25.

C = Combined counts of metaphases, averaging 31.7 grains, range 26-39.

TABLE XII

Grain counts over metaphases of lymphocytes incubated for the first 50 hours of culture with H^3-arginine.

Chromosome	Group	Observed	Expected	Chi-Square
1	A	42.0	41.8	0.001
	B	19.0	17.3	0.190
	C	23.0	24.5	0.100
2	A	51.0	38.9	4.090*
	B	23.0	16.1	3.220
	C	28.0	22.8	1.280
3	A	23.0	33.0	3.220
	B	12.0	13.7	0.230
	C	11.0	19.3	3.900*
4-5	A	58.0	53.5	0.430
	B	26.0	22.1	0.770
	C	32.0	31.4	0.010
13-15	A	45.0	48.6	0.300
	B	21.0	20.1	0.040
	C	24.0	28.5	0.790
19-20	A	9.0	22.4	21.640*
	B	2.0	9.2	5.900*
	C	7.0	13.2	2.980
21-22	A	18.0	15.1	0.580
	B	7.0	6.2	0.100
	C	11.0	8.9	0.570

The metaphases in this experiment were grouped according to grain count in order to detect any shift from random to nonrandom distribution. In chromosomes #1, #4-5, #13-15 and #21-22 counts do not deviate significantly from the expected on the assumption of a random and uniform labeling pattern. Where all metaphases are combined for #2, significance is obtained with the observed count exceeding the expected. Comparing chi-square values of the sub-groups, it would appear that most of the departure from random is due to the low-count group indicating that these are early-incorporating chromosomes. In the high-count group which was exposed longer to isotope, the deviation is less extreme. Apparently then chromosome #2 is early-labeling with respect to the rest of the complement, and the higher observed grain count is not due to a natural excess of arginine in the chromosome structure.

In chromosome #3 the observed counts are significantly lower than the expected in the high count group, indicating that these chromosomes lag behind the rest in beginning incorporation. However the possibility of a low concentration of arginine is not ruled out because high-count metaphases of random grains distribution were not observed in this series. The lack of significance in the low group may be due to background or cytoplasmic disintegrations which, though few in this experiment, should approximate random distribution.

Group #19-20 appears to start labeling later than

other groups as evidenced by a significant chi-square value in the low group and lack of significance in the high.

H^3-lysine

In the first experiment, lymphocytes were exposed to isotope for the first 24 hours. The 22 metaphases showed little label, averaging 9.0 grains with a range of 5 - 14, and were not sub-divided as to count. The results appear in Table XIII.

TABLE XIII

Grain counts over metaphases of lymphocytes incubated for the first 24 hours with H^3-lysine.

Chromosome Group	Observed	Expected	Chi-Square
1	10.0	17.1	3.230
2	18.0	15.9	0.300
3	5.0	13.5	5.740*
4-5	25.0	21.9	0.490
13-15	17.0	19.9	0.470
19-20	8.0	9.2	0.160
21-22	2.0	6.2	2.930

As with H^3-arginine, chromosome #3 has less label than expected. This implies that the rest of the complement has begun incorporation even though the grain count is very low, or the group may be deficient in lysine.

This experiment was repeated, incubating with isotope for 60 hours in order to obtain a wide range of counts. The results are presented in Table IV.

A = Combined counts of all 30 metaphases, averaging 16.1 grains.

B = Combined counts of metaphases, averaging 10.8 grains, range 7-15.

C = Combined counts of metaphases, averaging 20.8 grains, range 16-27.

TABLE XIV

Grain counts over metaphases of lymphocytes incubated for the first 60 hours with H^3-lysine.

Chromosome	Group	Observed	Expected	Chi-Square
1.	A	38.0	41.4	0.150
	B	13.0	13.9	0.060
	C	25.0	26.8	0.130
2	A	31.0	38.6	1.630
	B	11.0	13.0	1.340
	C	20.0	25.0	1.560
3	A	19.0	32.8	6.220*
	B	6.0	11.0	1.550
	C	13.0	21.2	3.400
4-5	A	55.0	53.0	0.090
	B	19.0	17.8	0.090
	C	36.0	34.3	0.090
13-15	A	65.0	48.2	6.500*
	B	25.0	16.2	4.180*
	C	40.0	31.2	2.760
19-20	A	4.0	22.2	15.620*
	B	3.0	7.4	2.740
	C	1.0	14.4	13.060*
21-22	A	6.0	14.9	5.480*
	B	4.0	5.2	0.200
	C	2.0	9.7	6.310*

As in the first experiment, chromosome #3 has a lower grain count than expected. Most of the deviation is contributed by the high-count group. These chromosomes probably commence labeling later, considering the H^3-arginine experiment. However, metaphases with high random counts were unavailable. Similar reasoning applies to #19-20 and #21-22 groups. It is noteworthy that the #21-22 group also had a low observed count in the first experiment and that the deviation was considerable, though not significant.

Group #13-15 would appear to begin labeling early, with a significantly higher count tnan expected in the low group, followed by non-significance in the high class.

Little chromosomal protein incorporation is evident until after 48 hours' incubation with either isotope. This is interesting in view of the early nuclear incorporation observed in the earlier experiments. If chromosomal proteins are synthesized at this time they are not transferred to the chromosomes until much later. This is in agreement with Cave (1966) who observed little synthesis of acidic proteins during G_1, with an increase during the S phase. As mentioned earlier, DNA synthesis commences about 24 hours after phytohemagglutinin stimulation.

Late addition of isotope H^3-arginine

Isotope was added to the culture during the last 4 hours before chromosome preparations were made. Grain counts appear in Table XV.

A = Combined counts of all 46 metaphases averaging 71.2 grains.

B = Combined counts of metaphases averaging 52.8 grains, range 30-70.

C = Combined counts of metaphases averaging 93.2 grains, range 71-144.

TABLE XV

Grain counts over metaphases of lymphocytes incubated for the last 4 hours with H^3-arginine.

Chromosome	Group	Observed	Expected	Chi-Square
1	A	271.0	281.8	0.450
	B	114.0	113.4	0.001
	C	157.0	168.4	0.840
2	A	272.0	262.2	0.400
	B	116.0	105.5	1.130
	C	156.0	156.6	0.002
3	A	182.0	222.8	8.010*
	B	68.0	89.7	5.650*
	C	114.0	133.1	2.940
4-5	A	403.0	360.5	5.630*
	B	174.0	145.1	6.470*
	C	229.0	215.4	0.970
13-15	A	330.0	327.7	0.020
	B	129.0	131.9	0.060
	C	201.0	195.8	0.150
19-20	A	127.0	150.7	3.910*
	B	53.0	60.7	1.030
	C	74.0	90.1	3.020
21-22	A	119.0	101.6	2.990
	B	52.0	40.9	3.110
	C	67.0	60.7	0.670

Chromosome #3 appears to finish labeling significantly earlier than the rest of the complement. That the low grain counts are not due to an arginine deficiency is indicated by the transition from significance in the low group to random distribution in the high. (In these studies it is assumed that metaphases with lower counts were near the end of protein incorporation when isotope was added.)

Group #4-5 on the contrary is late in finishing labeling with significantly higher counts than expected in the low group and no statistical significance in the high-count class.

The #19-20 class counts are significantly lower than expected when all metaphases are considered. The greater deviation is contributed by the high-count class, so it is uncertain whether protein synthesis was completed earlier or whether the arginine content of these chromosomes is low.

This experiment was repeated and the results obtained are as indicated in Table XVI.

A = Combined counts of all 21 metaphases averaging 37.9 grains.

B = Combined counts of metaphases with grain counts of 30-50, averaging 29.2.

C = Combined counts of metaphases with grain counts of 60-107, averaging 77.0.

TABLE XVI

Grain counts over metaphases of lymphocytes incubated for the last 4 hours with H^3-arginine.

Chromosome	Group	Observed	Expected	Chi-Square
1	A	73.0	68.4	0.340
	B	40.0	35.3	0.680
	C	33.0	33.1	0.001
2	A	58.0	63.7	0.550
	B	24.0	32.9	2.620
	C	34.0	30.8	0.360
3	A	47.0	54.1	1.000
	B	25.0	27.9	0.320
	C	22.0	26.2	0.720
4-5	A	112.0	87.6	7.640*
	B	59.0	45.2	4.730*
	C	53.0	42.4	2.980
13-15	A	78.0	79.6	0.030
	B	37.0	41.1	0.450
	C	41.0	38.5	0.180
19-20	A	31.0	36.6	0.900
	B	13.0	18.9	1.930
	C	18.0	17.7	0.005
21-22	A	24.0	24.7	0.030
	B	14.0	12.7	0.130
	C	10.0	11.9	0.310

Again group #4-5 appears to be late-labeling, but #3 was not significantly early although the observed counts are lower than expected. The #19-20 class follows the same trend as in the first experiment but without statistical significance.

H^3-lysine

Isotope was added to cultures at various periods before chromosome preparation in 4 experiments. The results of the first 3 appear in Table XVII. The metaphases were not sub-grouped as to grain count because of insufficient range and number of counts.

A = Combined counts of 69 metaphases, average 6.9 grains, range 2-17, isotope treatment for last 4 hours.

B = Combined counts of 18 metaphases, average 17.9 grains, range 12-30, isotope treatment for last 8 hours.

C = Combined counts of 15 metaphases averaging 15.7 grains, range 11-22, isotope treatment for last 10 hours.

TABLE XVII

Grain counts over metaphases of lymphocytes incubated with H^3-lysine for several intervals near the end of the culture period.

Chromosome	Group	Observed	Expected	Chi-Square
1	A	39.0	40.9	0.100
	B	32.0	27.8	0.690
	C	22.0	17.5	1.270
2	A	27.0	38.1	3.510
	B	17.0	25.8	3.260
	C	12.0	16.3	1.230
3	A	14.0	32.4	11.210*
	B	20.0	22.0	0.180
	C	6.0	13.9	4.820*
4-5	A	61.0	52.4	1.580
	B	35.0	35.5	.020
	C	27.0	22.4	1.060
13-15	A	63.0	47.6	5.530*
	B	31.0	32.3	.050
	C	23.0	20.4	.330
19-20	A	3.0	21.9	17.100*
	B	7.0	14.8	4.310*
	C	2.0	9.4	6.100*
21-22	A	15.0	14.7	0.010
	B	7.0	10.0	0.930
	C	5.0	6.3	0.280

In all 3 experiments, chromosomes #1, #2, #4-5 and #21-22 seem to be incorporating label in a random, uniform fashion as compared to the rest of the complement. This was also true of chromosomes #1, #2 and #21-22 in the late H^3-arginine experiments.

Chromosome #3 is again lower in expected grain count as in the late H^3-arginine trial of Table XV. So is group #19-20.

Only the "A" experiment indicates late-labeling for the #13-15 group. This study had a much lower average grain count than did "B" or "C" and considered together, these experiments indicate a shift to random grain distribution with an increase in exposure time to isotope.

In the fourth experiment sufficient metaphases were obtained to sub-group on the basis of grain count. In this trial H^3-lysine was included for the last 6 hours of culture. The results appear in Table XVIII.

A = Combined counts of all 37 metaphases averaging 18.3 grains.

B = Combined counts of metaphases averaging 13.2 grains, range 9-15.

C = Combined counts of metaphases averaging 21.7 grains, range 16-36.

TABLE XVIII

Grain counts over metaphases of lymphocytes incubated for the last 6 hours with H^3-lysine.

Chromosome	Group	Observed	Expected	Chi-Square
1	A	63.0	58.1	0.450
	B	19.0	17.0	0.260
	C	44.0	41.1	0.220
2	A	57.0	54.1	0.170
	B	19.0	15.8	0.700
	C	38.0	38.2	0.001
3	A	33.0	46.0	3.940*
	B	4.0	13.5	7.170*
	C	29.0	32.5	0.410
4-5	A	82.0	74.4	0.880
	B	22.0	21.8	0.001
	C	60.0	52.5	1.200
13-15	A	74.0	67.6	0.670
	B	21.0	19.8	0.080
	C	53.0	47.8	0.620
19-20	A	18.0	31.1	5.780*
	B	7.0	9.1	0.500
	C	11.0	22.0	5.760*
21-22	A	17.0	21.0	0.780
	B	4.0	6.1	0.740
	C	13.0	14.8	0.220

Again chromosomes #1, #2, #4-5 and #21-22 appear to be labeling in a random fashion. Group #13-15 also does not differ significantly from the expected, which agrees with the results in the previous 2 experiments having similar averages. Chromosome #3 appears to be earlier in completing incorporation, with a shift to tandom distribution in the high-count class. Group #19-20 is significantly lower in grain count than ex½ected, indicating either a deficiency in lysine as compared to the rest of the complement, or that these chromosomes finish labeling before the rest.

A comparison of the 2 isotopes reveals some interesting relationships. In the early-labeling experiments, chromosomes #1 and #4-5 appear to incorporate protein at the same time as the rest of the complement regardless of amino acid. Chromosomes #3 and #19-20 have consistently low counts for both isotopes. However the labeling patterns do not agree for #2, #13-15 or #21-22. In the late-labeling experiments, #1, #2 and #21-22 do not deviate from the expected for either isotope, and #3 and #19-20 are consistently low in count. The only difference in isotopes is evident in the labeling pattern of group #4-5. The functional significance of the difference in amino acid behaviour is unknown. It may reflect a difference in labeling pattern between the arginine-rich and lysine-rich histone fractions.

It is somewhat remarkable that chromosomes #3 and #19-20

both begin protein incorporation after the other chromosomes and also complete it ahead of them.

There is some correspondence between my results and those obtained by investigators studying the pattern of DNA synthesis. Kikuchi and Sandberg (1964) found that #22 was late in beginning H^3-thymidine incorporation and Mukherjee and Sinha (1965) present similar results for the #19-20 group. I have observed a similar pattern of protein synthesis for these groups. Gilbert *et al*. (1962), Bader *et al*. (1963) and Bianchi and de Bianchi (1965) showed that members of these groups were also among the first to complete labeling with H^3-thymidine. I have found that #19-20 was first to finish incorporation with both amino acids. In the H^3-arginine experiments group #4-5 completed labeling after the rest of the complement; members of this group were also shown to be late-labeling with respect to H^3-thymidine by Kikuchi and Sandberg (1964) and by German (1962). Agreement in patterns of labeling has already been reported by Prescott and Stone (1965) for hamster chromosomes, using H^3-amino acids and H^3-thymidine. However in order to make valid comparisons, the pattern of DNA synthesis should be investigated by the same statistical approach as used for the present study.

It must be re-emphasized that the type of protein being incorporated in this study is not defined. Certainly all the acidic proteins should have been preserved *in situ*. The fate of the chromosomally-associated enzyme systems is

unknown. Undoubtedly most, if not all, of the histone has been lost, as shown by the fixation trials with formalin and trichloroacetic acid and by Busch et al. (1964) and De (1961). Although the former claim that no further protein was removed by 0.25 N hydrochloric acid (the traditional histone solvent), I have found that my acetic-alcohol preparations do stain faintly for histones. Experiments are now in progress to develop a fixative combining formalin and trichloroacetic acid in an effort to conserve maximum chromosomal protein. Also labeling trials with H^3-tryptophan are being undertaken to assess the distribution of non-histone protein.

It was expected that some distinctive labeling pattern would emerge for at least one X chromosome. In 5 or 6 metaphases in one of the late-labeling H^3-arginine studies a large, heavily labeled chromosome in the #6-X-12 group was observed. However the identity of this chromosome was uncertain because of the technique employed. The chromosome groups studied in this work were chosen for ease of identification through the microscope without the aid of photography. Analyses of the rest of the complement, including the X chromosome, should only be performed by photographing the metaphase before application of the emulsion and later re-photographing the same metaphase with accompanying silver grains. These chromosomes can only be identified by cutting out the photographs and grouping according to size and centromere position. Future labeling

studies of the X-chromosome may indeed reveal a late labeling pattern, particularly if a better fixative is employed. The type of histone present on this chromosome may be responsible for its genetic inactivity and may serve to condense it during interphase. Frenster et al. (1963) have shown that in the interphase calf thymus lymphocyte condensed chromatin is associated with lysine-rich histone. If this fraction is associated with the inert X-chromosome it may be identifiable by labeling pattern.

In general, this study is interpreted as preliminary evidence for a consistent pattern of protein incorporation into the human lymphocyte chromosomes. The arbitrary grouping of metaphases counts may have resulted in loss of precision. In the future the use of synchronized cultures should overcome this difficulty. Detailed studies of individual chromosomes may reveal distinctive labeling patterns which would aid in identification. The study of protein incorporation into centromeres, telomeres and secondary constrictions may elucidate the structure and function of these loci. The importance of normal protein synthesis in ensuring separation of homologues and normal spindle behaviour has been demonstrated by Kemp (1964) and it may be that normal chromosomal protein synthesis can be observed in cancer chromosomes. Therefore these investigations will be extended to those metaphases of lymphatic leukemia which are normal with respect to gross chromosome morphology.

SUMMARY AND CONCLUSIONS

1. Exposure to acetic-ethanol for varying periods up to 49 hours resulted in significant loss of label from the chromosomes in one of 2 experiments with H^3-lysine. No protein loss was noted in cultures incubated with H^3-arginine. It is suggested that the decrease in H^3-lysine label may reflect removal of histone, possibly the lysine-rich fraction.

2. Aqueous trichloroacetic-ethanol was found to preserve more chromosomal protein than acetic-ethanol, but only when employed at 0-4°C. Formalin pretreatment prior to fixation by acetic-ethanol was also observed to increase grain counts over the chromosomes. Evidently acetic acid does remove protein, probably histone, as claimed by Busch et al. (1964) and De (1961). It is hoped that a fixative combining trichloroacetic acid and formalin pretreatment can be developed for chromosomes preparations to be used in studies of total protein incorporation.

3. As a guide in determining when isotope should first be added in the early-labeling experiments, phytohemagglutinin-stimulated lymphocytes were evaluated for initial incorporation of isotope. Significant uptake of H^3-arginine was observed after 4 hours' incubation and after 6 hours with H^3-lysine. Cytoplasmic incorporation became significant somewhat later for both isotopes. Even though early nuclear incorporation of amino acids was apparent, it was found that to obtain labeled chromosomes, cultures should be incubated

with the isotopes for the first 24-48 hours.

4. In the early labeling-experiments it was found that with H^3-arginine chromosomes #3 and #19-20 had fewer grains than could be expected on the basis of random group labeling with respect to time. This was interpreted as probably being due to late commencement of chromosomal protein incorporation. Chromosomes #1, #4-5, #13-15 and #21-22 did not appear to be either unduly early or late in beginning incorporation. In cultures incubated with H^3-lysine, chromosomes #3 and #19-20 also appeared to be late in starting, and #21-22 had a significantly lower grain count than expected. Group #13-15 was observed to begin incorporation earlier than the rest of the complement.

5. In the late labeling experiments with H^3-arginine, chromosome #3 appeared to finish protein incorporation first. Group #19-20 also had significantly fewer grains. Group #4-5 was last to complete labeling; chromosomes #1, #2, #13-15 and #21-22 did not differ significantly from the rest of the complement. In the H^3-lysine experiments chromosomes #3 and #19-20 finished early while chromosomes #1, #2, #4-5 and #21-22 were neither significantly earlier or later than the other chromosomes.

6. As the type of protein studied in these experiments is undefined, this investigation will be repeated with fixatives preserving total protein and with H^3-tryptophan which labels non-histone protein.

7. This study will also be repeated with other individuals, both male and female, to determine whether the patterns observed are simply a peculiarity of the one female investigated.

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